ORIGINAL ARTICLE

Properties of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in red blood cell concentrate of different ABO groups during 30-day storage at 4 °C

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Objective The present study aimed to investigate the psychrophilic properties of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* as contaminants of donated blood.

Methods Bags with red blood cell concentrates (RBCCs) of A, B, and AB blood groups were inoculated with a bacterial suspension of *Y. enterocolitica* (0 : 3 and 0 : 8) and *Y. pseudotuberculosis* (serovars I and III) and stored at 4 °C for 30 days. Bacterial growth was measured at selected intervals after inoculation. Initial strains and their subcultures (isolated after 30 days' incubation at 4 °C) were tested for glycolytic activity and susceptibility to the bactericidal action of human serum.

Results It was found that all strains grew well in the RBCCs of A, B, and AB groups. After incubation at 4 °C they increased their glycolytic activity and became more sensitive to the killing ability of human serum.

Conclusions The prolonged storage of contaminated *Y. enterocolitica* and *Y. pseudotuberculosis* RBCCs at 4 °C induces bacterial multiplication to high levels and stimulates glycolytic activity of bacterial cells.

Keywords ABO blood groups, growth, glycolysis, susceptibility to serum, *Y. enterocolitica*, *Y. pseudotuberculosis*

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INTRODUCTION

Blood transfusion sometimes initiates transmission of pathogenic bacteria, which increases the risk of bacteremia and septicemia. Transfusion of *Yersinia enterocolitica*-infected blood is rare, but fatal complications during blood transfusion have been reported [1].

The organisms probably originate from bacteremia in the donor and can subsequently multiply at low temperatures [2]. *Y. enterocolitica* grows well at 4 °C and in the presence of dextrose and iron. If blood is contaminated at the time of collection, storage of the red blood cells at 4 °C provides an ideal environment for bacterial growth and endotoxin production [3]. The aim of this study was to investigate the effect of long cold storage (4 °C) on bacterial growth and some properties of *Y. entercolitica* and *Y. pseudotuberculosis* in donated red blood cell concentrates (RBCCs) of different ABO groups.

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MATERIALS AND METHODS

Bacterial strains

The strains used in this study were *Y. enterocolitica* 201/86, serovar 0 : 3, biovar 4 (plasmid-bearing); *Y. enterocolitica* 653, serovar 0 : 8, biovar 1B (plasmid-bearing); *Y. pseudotuberculosis* 32981, serovar I (plasmid-bearing); and *Y. pseudotuberculosis* Mollaret, serovar III (plasmidless). The strains of *Y. enterocolitica* were a kind gift of Prof. G. Kapperud (Norway) and were obtained from patients. The strains of *Y. pseudotuberculosis* were isolated from animals and kindly provided by Prof. H. Mollaret (France). A suspension of the test microorganisms was prepared from an overnight culture on tryptic soy agar (Difco, USA), diluted to 10^3 cells/mL in saline and then used to inoculate the RBCCs.

Blood processing and inoculation

The freshly donated blood from groups A, B, and AB was primarily processed in the regional Blood Bank Center in Sofia. Red blood cells were separated in bags by standard methods. The bags were inoculated with bacterial suspension of strains *Y. enterocolitica* 201/86, *Y. enterocolitica* 653, *Y. pseudotuberculosis*

32981, and *Y. pseudotuberculosis* Mollaret, so that the final bacterial concentration in each bag was 10^2 cells/mL for each strain. All bags were incubated at 4 °C and stored for 30 days. There was one non-inoculated control bag from each ABO blood group (A, B, and AB). During storage, samples were collected periodically (at 1, 3, 7, 15 and 30 days) and colony counts of serial dilutions were performed by plating on tryptic soy agar (Difco). Initial strains and their subcultures (isolated after 30 days of incubation at 4 °C) were tested for glycolytic activity and susceptibility to the bactericidal action of human serum.

Glycolytic activity of bacterial cells

Glycolysis was assayed essentially as described earlier by Radoucheva et al. [4]. One milliliter of bacterial suspension with a density of 2×10^7 cells/mL was added to a reaction mixture containing the following components in 2.5 mL: 80 µmol of Tris buffer (pH 7.2), 60 µmol of glucose and 25 µmol of KCN. After a 2 h incubation at 37 °C without shaking, 0.5 mL (for each sample) of 30% trichloracetic acid was added. The amount of lactic acid was determined in protein-free supernatant. In the presence of sulfuric acid and β-hydroxy-biphenil, the lactic acid was converted to a chromophore and absorption measured at $\lambda \approx 540$ nm in a Zeiss Spekol model 11 spectrophotometer (Zeiss, Jena, Germany). The results were expressed as µg of lactic acid released by 2×10^7 cells in 2 h.

Serum bactericidal assay

The serum bactericidal assay was carried out according to the method of Pai and DeStephano [5]. Serial twofold dilutions of human test serum were performed. Overnight cultures of *Y. enterocolitica* 201/86, *Y. enterocolitica* 653, *Y. pseudotuberculosis* 32981, and *Y. pseudotuberculosis* Mollaret were rinsed twice and suspended in saline to 5×10^3 colony-forming units (CFU)/mL. Bacteria were added to each serum dilution (in 100 µL volumes, 1 : 1 v/v). At time zero and after 2 h incubation at 37 °C, samples (0.1 mL) from each serum dilution were plated on meat peptone agar. CFU were counted after 48 h of incubation at 25 °C. The serum dilution ensuring 50% reduction of the initial bacterial inoculum during 120 min incubation of bacteria–serum mixture at 37 °C was accepted as the bactericidal titer.

Statistical analysis

The results were expressed as mean values ± standard deviation in order to assess the significance of differences within experiments. Student's *t*-test was used. Statistical significance was defined as $P < 0.01$. Experiments were repeated three times.

RESULTS

All *Y. enterocolitica* and *Y. pseudotuberculosis* strains demonstrated the ability to grow well for a period of 30 days in human RBCCs of A, B, and AB groups at 4 °C. After a lag period of at least 3 days, all strains entered a phase of exponential growth, which continued until the end of experiment (day 30). It was found that the final bacterial counts of *Y. enterocolitica* 0 : 3, i.e. $(4.5 \pm 0.5) \times 10^{11}$ in group A, $(3.4 \pm 0.4) \times 10^{10}$ in group B, and $(6.6 \pm 0.7) \times 10^{11}$ in group AB; and of *Y. enterocolitica* 0 : 8, i.e. $(1.3 \pm 0.2) \times 10^{11}$ in group A, $(2.1 \pm 0.3) \times 10^{10}$ in group B, and $(4.2 \pm 0.5) \times 10^{11}$ in group AB, were higher than those of *Y. pseudotuberculosis* serovar I, i.e. $(2.2 \pm 0.3) \times 10^{10}$ in group A, $(2.1 \pm 0.2) \times 10^{9}$ in group B, and $(4.9 \pm 0.6) \times 10^{10}$ in group AB; and of *Y. pseudotuberculosis* serovar III, i.e. $(4.2 \pm 0.6) \times 10^{9}$ in group A, $(1.3 \pm 0.4) \times 10^{8}$ in group B, and $(1.3 \pm 0.2) \times 10^{10}$ in group AB. Statistically significant correlations between blood group type (A, B, or AB) and growth capacity of strains were not found.

The results of glycolytic activity tests are summarized in Figure 1. After incubation at 4 °C in RBCCs, all strains increased their glycolytic activity. The strains of *Y. enterocolitica* were more active than those of *Y. pseudotuberculosis*. There were statistically significant differences in the values (at $P < 0.01$) between *Y. enterocolitica and Y. pseudotuberculosis*. The strains isolated after incubation in the RBCCs of blood group B showed high levels of glycolytic activity (*Y. enterocolitica*

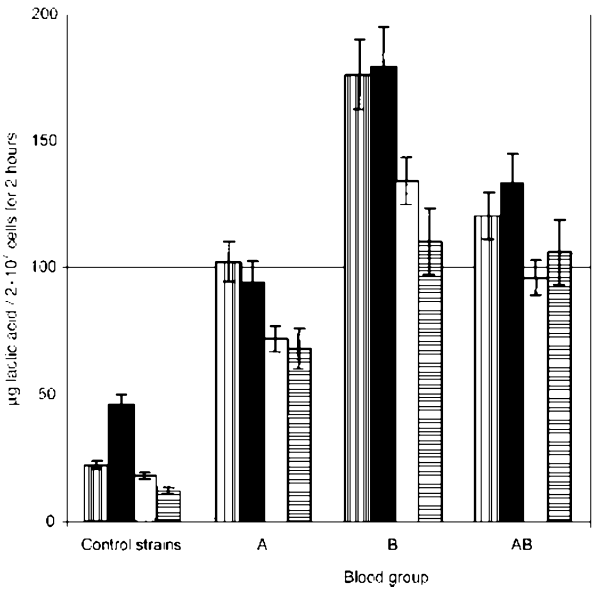

Figure 1 Glycolytic activity of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* after 30 days of incubation at 4 °C in blood bags with red cells concentrate. ▥ *Y. enterocolitica* 201/86 serovar 0 : 3; ■ *Y. enterocolitica* 653 serovar 0 : 8; □ *Y. pseudotuberculosis* 32981 serovar I; ▤ *Y. pseudotuberculosis* Mollaret servor III.

Table 1 Susceptibility of *Y. enterocolitica* and *Y. pseudotuberculosis,* isolated after 30 days of incubation in red blood cells concentrate at 4 °C to bactericidic activity of human serum

Strain	Initial titre (prior to incubation)	Titre after incubation in blood of Group:		
		A[a]	B[a]	AB[a]
Y. enterocolitica 201/86 serovar 0 : 3	8 ± 3.59[b]	64 ± 28.79[c]	32 ± 14.39	64 ± 24.19[c]
Y. enterocolitica 653 serovar 0 : 8	4 ± 1.51	16 ± 7.20	8 ± 3.60	16 ± 7.20
Y. pseudotuberculosis 32981 serovar I	4 ± 1.51	64 ± 12.09[c]	32 ± 14.40	64 ± 28.79[c]
Y. peudotuberculosis Mollaret serovar III	8 ± 3.60	128 ± 24.19[c]	64 ± 12.09[c]	128 ± 24.19[c]

[a]Blood group; [b]mean ± standard deviation serum titers; [c]significant differences from control strains ($P < 0.01$).

201/86 serovar 0 : 3, 176 ± 11 μg of lactic acid; *Y. enterocolitica* 653 serovar 0 : 8, 179 ± 16 μg; *Y. pseudotuberculosis* 32981 serovar I, 134 ± 19 μg of lactic acid; *Y. pseudotuberculosis* Mollaret serovar III, 110 ± 12 μg). The strains incubated in blood group A were the least active.

The strains isolated after incubation in blood cell concentrates at 4 °C were tested for susceptibility to the bactericidal action of human serum. As presented in Table 1, all strains showed a trend to increase their susceptibility to the killing ability of the test serum. The strains of *Y. pseudotuberculosis* were more sensitive. The bactericidal titer for *Y. enterocolitica* 201/86 serovar 0 : 3 increased from 1 : 8 (initial strain) to 1 : 64 (three times) in RBCCs of group A, to 1 : 32 (two times) in RBCCs of group B, and to 1 : 64 (three times) in RBCCs of group AB. The bactericidal titer for *Y. enterocolitica* 653 serovar 0 : 8 increased from 1 : 4 (initial strain) to 1 : 16 (two times) in RBCCs of group A, to 1 : 8 (one time) in RBCCs of group B, and to 1 : 16 (two times) in RBCCs of group AB. The bactericidal titer for *Y. pseudotuberculosis* 32981 serovar I increased from 1 : 4 (initial strain) to 1 : 64 (four times) in RBCCs of group A, to 1 : 32 (three times) in RBCCs of group B, and to 1 : 64 (four times) in RBCCs of group AB. The results for *Y. pseudotuberculosis* Mollaret serovar III were similar.

DISCUSSION

Contamination of donated blood has been reported in several cases of blood transfusion. Among the bacterial strains, *Y. enterocolitica* has emerged as a significant cause of transfusion-associated bacteremia and mortality [1,6]. Tipple et al. [3] investigated seven cases of transfusion-associated *Y. enterocolitica* sepsis: four were caused by organisms of serotype 0 : 3, and one each was caused by organisms of serovar 0 : 1, 2, 3, 0 : 5, 27, and 0 : 20. Review of the cases presented by Bottone [6] reveals that serogroup 0 : 3 *Y. enterocolitica* accounts for 15 (55%) of the 27 episodes of transfusion-acquired *Y. enterocolitica*, followed by serogroups 0 : 9, 0 : 5, 27, 0 : 123, and 0 : 20. Unlike most other contaminants of blood components, *Y. enterocolitica* can actively multiply at refrigerator temperatures (4 °C) during blood storage and can cause severe reactions in recipients. Our previous study demonstrated that, in contrast to *Salmonella typhimurium* and *Stophylococcus aureus*, which showed a trend to die out during a 30-day incubation period at 4 °C in RBCCs, *Y. entercolitica* and *Y. pseudotuberculosis* multiplied rapidly to large numbers [7]. It is supposed that *Y. entercolitica* may persist in human tissues and can enter donated blood as a result of transient bacteremia [8].

In the present study it was observed that all strains were capable of growth at 4 °C in the RBCCs of A, B, and AB groups. Results of the glycolytic activity studies indicated that glycolysis increased in all tested strains. Incubation at a low temperature in human RBCCs in our experiment had a positive effect on the catabolic metabolism of all strains. It is known that the level of glycolytic activity is accepted as an indicator for the energy metabolism of organisms. Obviously, the enhanced catabolism of glucose taking place during 'cold' cultivation of *Y. enterocolitica* and *Y. pseudotuberculosis* ensures the increased physiological needs of bacterial cells and appears to be a part of the adaptive metabolic manifestation in these bacteria. Somov and Varvashevich [9] suggest that low temperature, in combination with other definite factors, triggers the activity of certain isoenzymes in *Y. pseudotuberculosis* that function only at low temperatures. In fact, there are no data on *Y. pseudotuberculosis* as a contaminant of blood products. The effect of low positive temperature on the growth capacity of *Y. enterocolitica* and *Y. pseudotuberculosis* has been widely studied [10–13]. Long periods of incubation at low positive temperatures led to a higher degree of adaptation [14]. Increased phenotypic tolerance to cold is accompanied by the synthesis of proteins involved in different functions [4]. It is known that *Y. enterocolitica and Y. pseudotuberculosis* can accumulate Fe^{3+} and can use hemin as a sole source of iron. They possess an efficient cell-bound transport system for Fe^{3+} [15]. Tipple et al. [3] suggest that *Y. enterocolitica* growth in human blood may be enhanced by iron liberated from aging erythrocytes and also by saline–dextrose solution, which is used for the collection and resuspension of erythrocytes.

Experiments involving serum sensitivity tests showed that all strains increased their susceptibility to the bactericidal action of serum after incubation in RBCCs at 4 °C for 30 days in different grades. Resistance to serum bactericidal activity of

yersinial cells is manifested at 37 °C and correlated with presence of two outer membrane proteins – YadA (plasmid encoded) and Ail or Inv (chromosomally encoded). Yersinial cells growing at lower temperatures have low copy numbers of these proteins [6,16,17]. So, at 4 °C, the plasmid is no longer expressed, synthesis of these proteins is down-regulated, and the bacterial cells become sensitive to serum [18].

We did not find any significant influence of ABO blood type on the growth capacity of *Yersinia* strains. Interestingly, we noted that, after incubation, isolated strains in blood group B demonstrated the most activated glycolytic activity. It could be suggested that the regulation of adaptive metabolism in *Y. enterocolitica* and *Y. pseudotuberculosis* is a complex process depending at least on specific species and strain properties, and on environmental (host) factors.

CONCLUSION

In conclusion, *Y. enterocolitica* and *Y. pseudotuberculosis* demonstrate adequate metabolic reorganization, successful adaptation and multiplication to high levels during long storage in donated RBCCs at 4 °C, which makes them particularly dangerous as contaminants.

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